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Huntington's disease (HD) is a neurodegenerative condition characterized by a loss of projection neurons in the striatum. Although various hypotheses have been proposed to explain the mechanisms that underlie the striatal neuronal death, excitotoxicity still deserves major interest. Recent findings indicate that changes in the genotype of the kainate receptor subunit, GluR6, are associated with variation in the age of onset of HD, which implicates the kainate receptors in the pathogenesis of HD. The rationale of this project is that pre-synaptic kainate receptors control the release of glutamate from cortical or thalamic terminals, and that an abnormal regulation of these receptors is involved in the death of striatal neurons in HD. We, therefore, propose to use state-of-the-art electron microscope techniques to test a series of hypotheses that will help to elucidate the localization and understand better the role of kainate receptors in the primate striatum. The results of these studies will provide a strong basis for studying the potential mechanisms by which these receptors participate in the death of striatofugal neurons in HD. Moreover, they will help the development of novel therapeutic strategies aimed at targeting pre-synaptic kainate receptors in HD and other basal ganglia disorders.

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INTRODUCTION

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder characterized by the death of striatal neurons. Chorea is the most common involuntary movement in patients who suffer of HD. This could be combined with cognitive and memory deficits at a later stage of the disease. The HD mutation was identified in 1993 as an unstable expansion of CAG (trinucleotide) repeats on the gene which encodes the protein "Huntingtin" on chromosome 4. In more than 60% of HD patients, there is a high degree of inverse correlation between the number of CAG repeats and the age of onset of the disease or degree of striatal degeneration (Vonsattel and DiFiglia, 1998). However, about 15% of the HD cases of which the age of onset cannot be explained by the CAG repeats, were found to have mutations in the gene encoding for the GluR6 subunit of the glutamatergic kainate receptor (Rubinztstein et al., 1997; MacDonald et al., 1999); which highlight the importance of those receptors in the pathogenesis of the striatum in HD. Although the existence of kainate receptors has long been established, little is known about their functions and distribution in the central nervous system. Previous data obtained in our laboratory showed that the kainate receptor subunits GluR6/7 are strikingly enriched in the monkey striatum but, in contrast to other ionotropic glutamate receptors which are found almost exclusively at postsynaptic sites, the GluR6/7 kainate receptor subunits are strongly expressed pre-synaptically in glutamatergic terminals (Charara et al., 1999).

Based on these data, the rationale of experiments proposed in this application was that altered functions of pre-synaptic kainate receptors, due to mutations of the GluR6 subunit gene, may induce excessive glutamate release in the striatum, thereby, excitotoxic cell death of striatal projection neurons in Huntington's disease. Before addressing such an issue, a prerequisite is to characterize in detail the synaptic localization of kainate receptors in the striatum. We, therefore, proposed to use a combination of various anatomical and immunocytochemical approaches at the electron microscopic level to elucidate the pattern of subcellular and subsynaptic localization of GluR6 and KA2 subunits of the kainate receptors in the monkey striatum.

BODY

- **SPECIFIC AIMS**

The original proposal comprised the following specific aims:

Hypothesis I: The GluR6/7 kainate receptor subunits are strongly expressed by cortical glutamatergic terminals in the monkey striatum.

Specific Aim #1: To elucidate the subsynaptic localization of GluR6/7 immunoreactivity in the striatum using immunoperoxidase and immunogold techniques at the electron microscope level.

Specific Aim #2: To demonstrate that GluR6/7-immunoreactive terminals arise from the cerebral cortex using a combination of tract-tracing techniques and pre-embedding immunogold methods.

Hypothesis II: The pre-synaptic kainate receptors are more frequently encountered in those regions of the striatum that are more sensitive to degeneration in HD.

Specific Aim #3: To compare the relative frequency of GluR6/7-immunoreactive terminals between the rostral and caudal portions of the putamen and between the tail, body and head of the caudate nucleus.

Hypothesis III: The terminals that express kainate receptor subunits form synaptic contacts preferentially with the "indirect D2-containing" striatofugal neurons which degenerate first in HD.

Specific Aim #4: To compare the relative frequency of synaptic contacts established by GluR6/7-immunoreactive terminals with "direct D1-containing" and "indirect D2-containing" striatofugal neurons.

Hypothesis IV: The GluR6/7 and KA2 kainate receptor subunits are expressed at pre-and post-synaptic sites in the striatum.

Specific Aim #5: To compare the subsynaptic localization of KA2 and GluR6/7 immunoreactivity in different regions of the striatum. .

Hypothesis V: The diffusion of glutamate from the synaptic cleft to pre-synaptic kainate receptors is controlled by glutamate transporters.

Specific Aim #6: To study the relationships between the glutamate transporters and the GluR6/7-immunoreactive terminals.

PROGRESS REPORT 2002-2003

As stated in our 2001-2002 progress report, specific aims 1,2,3 and 5 of this grant have been completed and the data have been published in various peer-reviewed manuscripts and presented in abstract forms at National and International meetings. This report also presented the current status of experiments for Specific aims #4 and #6 as well as a new series of data on the electron microscopic localization of kainate receptor subunits in the monkey globus pallidus.

In the following account, I will briefly outline the progress that has been made over the past year towards the achievement of these studies and discuss the relevance of the data obtained for a deeper understanding of the role of kainate receptors in basal ganglia physiology and pathophysiology of Huntington's disease and other related movement disorders.

I. Hypothesis 3-Specific Aim #4

The goal of these studies was to test the possibility that kainate receptor-containing glutamatergic terminals from synapses more frequently with so-called "indirect" striatofugal neurons that project to the external globus pallidus (GPe) than with the "direct" striatofugal neurons that project to the internal globus pallidus (GPi). The rationale of these studies being that "indirect" striatofugal neurons are more sensitive to degeneration than "direct" striatofugal neurons in Huntington's disease. If the gene that encodes for the GluR6 kainate receptor subunit is, indeed, affected in Huntington's disease (see above), one may speculate that the pre-synaptic kainate receptors expressed in glutamatergic terminals in the striatum may function abnormally leading to excessive glutamatergic transmission followed by excitotoxic degeneration of striatal neurons. Under these pathological conditions, tighter synaptic relationships between a specific population of striatal neurons and kainate receptor-containing terminals may underlie a differential degree of degeneration of the two populations of striatofugal neurons in Huntington's disease.

To reach the goal of these studies we used a combination of retrograde labeling of striatofugal neurons with the immunocytochemical localization of GluR6/7 receptor subunits in glutamatergic terminals in four monkeys. In two more animals we used D1 and D2 dopamine receptor labeling as selective markers of direct versus indirect striatofugal neurons.

In both series of experiments the results were the same i.e. we found that almost 70% of GluR6/7-containing terminals form synapses with the spines of "indirect" striatofugal neurons whereas less than 15% of them contact "direct" striatofugal neurons. *These findings provide strong evidence for a differential innervation of the two main populations of striatofugal neurons by kainate-containing glutamatergic terminals in the primate striatum. The preferential targeting of "indirect" striatal output neurons may underlie the differential sensitivity of direct versus indirect striatofugal neurons to neurodegeneration in Huntington patients with a mutation of the GluR6 gene.*

These data were presented in abstract forms and a manuscript is currently in preparation.

II. Hypothesis V-Specific Aim #6

The last specific aim of the proposed project is to elucidate the relationships between the glial glutamate transporter GLT-1 and glutamatergic terminals that express pre-synaptic kainate receptors, the rationale being that glutamate spillover, largely modulated by GLT-1, is the main source of activation of pre-synaptic kainate receptors. Since the two other glutamate transporters namely, EAAC1 and GLAST, are not significantly involved in controlling glutamate reuptake in the striatum, we will focus our interest towards GLT-1 considered as the main recipient for extracellular glutamate in the mammalian CNS. Therefore, if terminals that express pre-synaptic kainate receptors are tightly surrounded by processes of astrocytes that contain GLT-1, it strongly suggests that the most likely source of glutamate to activate these receptors is the neurotransmitter released by the parent terminals. On the other hand, if the kainate-containing terminals are not intimately related to GLT-1 processes, it would indicate that the extrasynaptic diffusion of glutamate from neighboring synapses may also have access to pre-synaptic kainate receptors, as was found for other glutamate receptor subtypes in various brain regions (Rusakov and Kullmann, 1998; Brasnjo and Otis, 2001; Danbolt, 2001).

In the last year progress report we discussed our preliminary data showing the expression of GLT-1 in a subset of kainate-containing glutamatergic terminals suggesting that GLT-1 may regulate activation of pre-synaptic kainate receptors through both glial and neuronal GLT-1-mediated glutamate reuptake in the primate striatum. These findings have now been confirmed in a larger group of animals. Data obtained so far indicate that 30-50% of GLT-1-containing glutamatergic terminals in the monkey striatum display GluR6/7 kainate receptor subunit immunoreactivity. It is noteworthy that the expression of GLT-1 in axon terminals is not unique to the striatum but has also been reported in other brain regions (Schmitt et al., 1996; Mennerick et al., 1998).

We have now undertaken the last part of this project, which aims at comparing the relative distribution and density of GLT-1-containing glial processes in relation to terminals that express or not pre-synaptic kainate receptors. To do so, we use a double post-embedding immunogold approach that labels GLT-1 and GluR6/7 immunoreactivity with two different sizes of gold particles. Once a GluR6/7-containing terminal is found, it is followed through serial ultrathin sections and the distance between the nearest plasma membrane-bound gold particles in astrocytes (ie GLT-1 immunoreactivity) and the midpoint of the postsynaptic specialization is measured for each synapse. The measurements made for kainate-containing terminals and

kainate-negative boutons are then compared statistically. Although still very preliminary, our data suggest that the average distance between the gold particles labeling for GLT-1 and synapses established by GluR6/7-positive boutons is 2.5 times shorter than that between GLT-1 gold particles labeling and asymmetric synapses that involve kainate-negative boutons. Data have so far been collected from a single monkey. Ongoing observations are in progress in two more animals. Our goal is to collect data from at least 30-50 terminals/monkey so that we can make firm conclusions on the specific relationships between glial glutamate transporters and kainate receptor-containing terminals in the primate striatum.

Part of these findings will be presented in abstract form at the next Society for Neuroscience meeting and some of our data on glutamate transporters in the striatum were recently published in a book chapter (Charara et al., 2002). A peer-reviewed manuscript should be prepared for publication during the coming year.

III. Pre- and post-synaptic Kainate Receptors in the Monkey Globus Pallidus

As mentioned in last year progress report, we have expanded our analysis of the subcellular and subsynaptic localization of kainate receptor subunits to the globus pallidus. In a first series of experiments, we have characterized the localization of GluR6/7 immunoreactivity in the monkey pallidum using the pre-embedding immunoperoxidase approach combined with the post-embedding immunogold localization of GABA. The main findings of this study is that kainate receptors are strongly expressed pre- and post-synaptically in both the internal (GPi) and external (GPe) segments of the monkey globus pallidus. At the pre-synaptic level, both GABAergic and non-GABAergic terminals display GluR6/7 immunoreactivity, which suggests that pre-synaptic kainate receptors can act as auto- or heteroreceptors in the monkey pallidum. These observations served as a basis for ongoing electrophysiological studies of the role of pre- and post-synaptic kainate receptors in regulating excitatory and inhibitory transmission in the globus pallidus (see below).

Results of this study have been presented at the last Society for Neuroscience meeting in Orlando and are currently in press for publication in Neuroscience (Kane-Jackson and Smith, 2002, 2003).

IV. Localization and Functions of Kainate Receptors in the Globus Pallidus

Based on the findings collected in monkeys showing the abundance of pre- and post-synaptic kainate receptors in GPe and GPi, we decided to undertake a series of in vitro slice electrophysiological studies to address the role of kainate receptor activation on pallidal neurons using whole cell patch clamp recording techniques in rats.

To make sure that the pattern of subcellular localization of kainate receptors described in monkeys is valid in rats, we carried out an electron microscopic analysis of GluR6/7 immunoreactivity in the rat globus pallidus. Although data have not yet been quantified, it appears that the main features of kainate receptor distribution in GPe and GPi are seen in the rodent GP, ie there is heavy GluR6/7 postsynaptic labeling associated with proximal and distal

dendritic shafts as well as pre-synaptic labeling in putative GABAergic and glutamatergic axon terminals (Jin and Smith, 2003).

We have made significant progress in testing the role of postsynaptic kainate receptor activation on GP neuronal activity. In brief, these studies are performed as follows: Fourteen- to 17 days old Sprague Dawley rats are used in these experiments. After decapitation, brains are removed and quickly submerged in the ice-cold oxygenated sucrose buffer. Coronal slices (300 μM) are made on a vibratome in ice-cold oxygenated sucrose buffer. Slices are then stored at room temperature in a chamber containing artificial cerebrospinal fluid (ACSF) at pH 7.35-7.45 with 95% O_2 , 5% CO_2 bubbling through it. A slice is then transferred to a recording chamber and perfused with room temperature oxygenated ACSF. GP neurons are visualized with a 40X water immersion lens using a Hoffman modulation contrast microscope. Whole cell electrodes are pulled on a vertical patch pipette puller and filled with an intracellular patch solution. Biocytin at 1% is included in the intracellular solution to view the morphology and location of GP neurons. The drugs used to activate kainate receptors (Kainate, domoate) and block AMPA receptors (SYM 2206, GYKI52466, NBQX) are bath applied. TTX (0.5 μM) and DAP5 (NMDA receptor antagonist-25 μM) are perfused at least 5 min before experiments. Data are acquired and analyzed using pCLAMP software. Results are presented as mean \pm S.D., and significance evaluated by Student's *t*-test.

Two main groups of GP neurons have been categorized electrophysiologically (Types I and II- Cooper and Stanford, 2000). The type II neurons account for almost 80-90% of the total population of GP neurons, thereby, represent the major subtype of neurons that will be examined in our study. These neurons possess two main cardinal features, both of which are recorded at the beginning of our experiments: (1) A sag in membrane potential during a hyperpolarizing current injection in current clamp that corresponds to a time- and voltage-dependent inward current I_h , (2) Anodal breaks after a hyperpolarizing step, suggesting the presence of a low-threshold-activated calcium current I_t . These neurons also show high input resistance and spontaneous activity at rest (Figure 1A). In an attempt to correlate the morphology and relative position of GP neurons with their electrophysiological profiles, recorded neurons are filled with biocytin (Figure 1B).

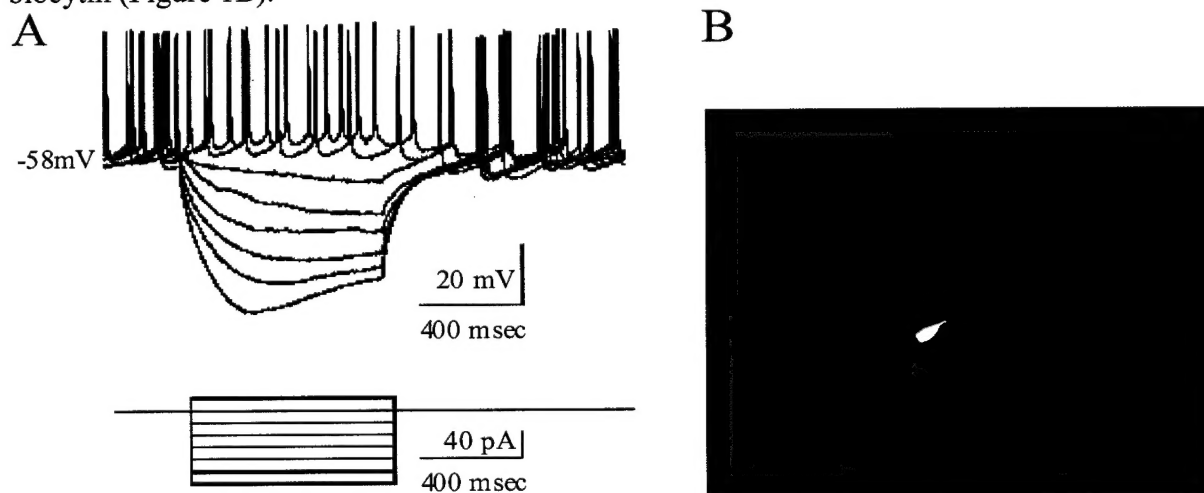


Figure 1: Electrophysiological (A) and morphological (B) characteristics of type II GP neurons

Figure 2 shows evidence for the expression of functional kainate receptors on GP neurons. Panel A illustrates inward currents evoked by $5\mu\text{M}$ kainate in two GP neurons in the presence of AMPAR antagonists SYM 2206 ($100\mu\text{M}$) and NBQX ($1\mu\text{M}$). Antagonists were bath applied for 3 min before exposure to kainate. TTX ($0.5\mu\text{M}$) was bath applied for at least 5 min before the beginning of all experiments. D-AP5 ($25\mu\text{M}$) was bath applied throughout the experiments. In panel B, we show the mean amplitude of inward current activated by $5\mu\text{M}$ kainate in the presence of SYM 2206 ($100\mu\text{M}$) (mean \pm S.D, $n=6$ cells) and NBQX ($1\mu\text{M}$) (mean \pm S.D, $n=7$ cells). These preliminary data demonstrate that kainate receptor activation induces inward current in rat GP neurons suggesting the presence of functional postsynaptic kainate receptors in GP.

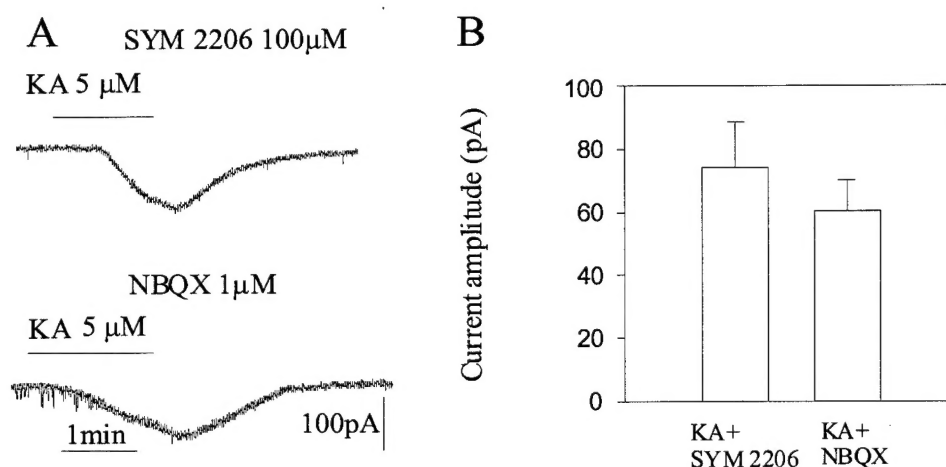


Figure 2: Functional Expression of Postsynaptic Kainate Receptors in rat GP

Further evidence for functional kainate receptors in GP are shown in figure 3. Panels A and B are current-clamp recordings from three GP neurons in the presence of TTX ($0.5\mu\text{M}$) and DAP5 ($25\mu\text{M}$). In the presence of AMPAR antagonists GYKI 52466 ($30\mu\text{M}$) (first trace) and NBQX ($1\mu\text{M}$) (second trace), bath application of kainate ($5\mu\text{M}$) depolarizes neuronal membrane of two cells. In panel B, bath application of Domoate ($0.5\mu\text{M}$), a kainate receptor agonist, depolarizes the cell in the presence of an AMPA receptors antagonist GYKI (52466) ($30\mu\text{M}$). Finally, panel C shows the mean amplitude of depolarization activated by $5\mu\text{M}$ kainate in the presence of GYKI 52466 ($30\mu\text{M}$) (mean \pm S.D, $n=6$ cells), NBQX ($1\mu\text{M}$) (mean \pm S.D, $n=10$ cells) and by $0.5\mu\text{M}$ domoate in the presence of GYKI 52466 ($30\mu\text{M}$) (mean \pm S.D, $n=5$ cells). These findings provide strong evidence that selective kainate receptor activation depolarizes GP

• Future Experiments

1. To examine if the response of GP neurons to kainate application is dose dependant. Five different doses of kainate ($0.1-10\mu\text{M}$) will be perfused in presence of AMPA antagonist (GYKI 52466 $30\mu\text{M}$) and dose response curve will be established.

2. To test if kainate receptors can be synaptically activated. The evoked EPSC will be recorded from GP neurons by stimulating the subthalamic nucleus (STN). If activation of kainate receptors contributes to evoked EPSC, kainate receptors-mediated EPSC should be recorded in presence of AMPA receptors antagonists.
3. To test if activation of kainate receptors modulate the glutamatergic synaptic transmission between STN and GP neurons. If application of kainate has an effect on the amplitude of evoked EPSC, we will perform experiments to determine if this is mediated by pre or postsynaptic mechanisms. To do so, we will test the effects of kainate receptor activation on paired-pulse facilitation of evoked EPSCs and on the frequency of spontaneous miniature EPSCs (mEPSCs).

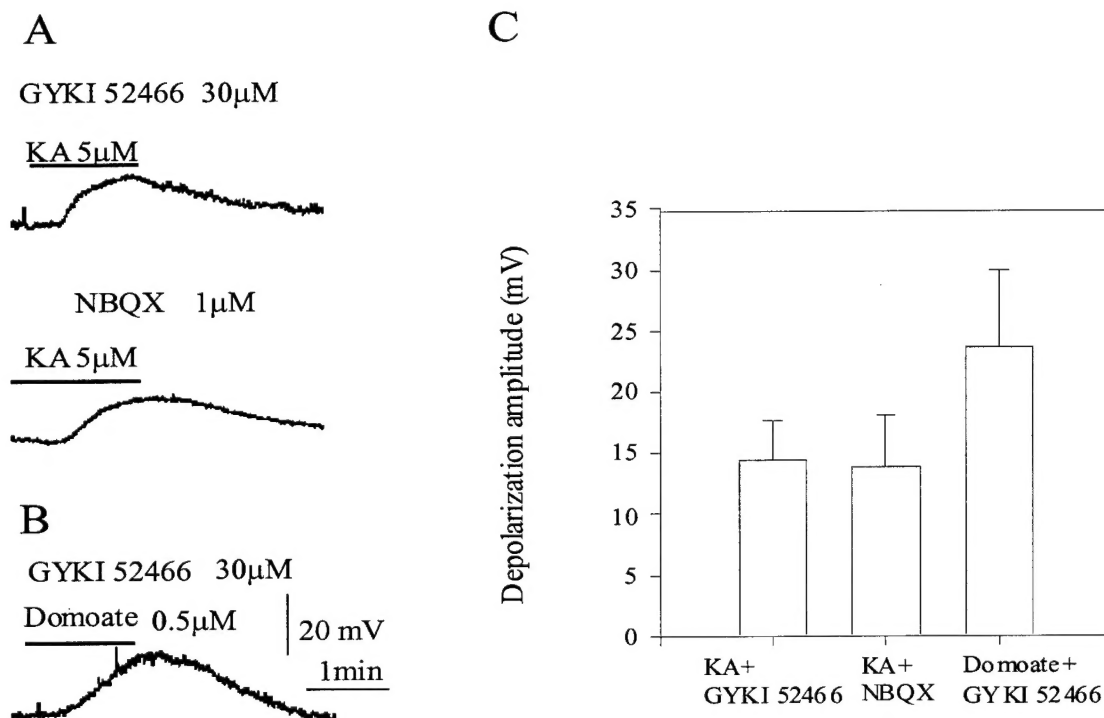


Figure 3: Kainate receptor activation depolarizes GP neurons

KEY RESEARCH ACCOMPLISHMENTS

The main findings obtained in this project over the past year are summarized as follows:

- Kainate receptor-containing glutamatergic terminals preferentially target the so-called "indirect" striatofugal neurons in monkeys. An abnormal regulation of pre-synaptic kainate receptors may therefore lead to increased glutamate release at these synapses, thereby, excitotoxic cell death. This could underlie the preferential degeneration of "indirect" striatofugal neurons in Huntington patients.

- The glutamate transporter GLT-1 is co-expressed with the GluR6/7 kainate receptor subunit in a subset of glutamatergic terminals in the monkey striatum. Preliminary data also suggest that GLT-1-containing glial processes are more abundant and closer to glutamatergic synapses established by kainate-containing terminals than those formed by kainate-negative boutons. These observations suggest that glutamate reuptake mechanisms through GLT-1 are critical in regulating the activation of pre-synaptic kainate auto-receptors in the striatum.
- Pre- and post-synaptic kainate receptors are strongly expressed in the monkey and rat globus pallidus. Pre-synaptic receptors are associated with both GABAergic and glutamatergic terminals suggesting that their activation may lead to auto- or hetero-regulation of neurotransmitters release.
- Kainate receptor activation evokes EPSC's that are insensitive to AMPA or NMDA receptor antagonists in rat GP neurons, which suggest the functional expression of postsynaptic kainate receptors in pallidal neurons.
- Kainate receptors activation depolarizes GP neurons in the presence of TTX and AMPA/NMDA receptor antagonists. The importance of these data regarding basal ganglia pathophysiology of various movement disorders including HD is the potential development of novel drugs that could selectively target GluR6-containing kainate receptors and modulate glutamatergic and GABAergic neurotransmission in the globus pallidus. It is still premature to speculate about a particular treatment strategy at this point without knowing the exact role pre-synaptic kainate receptors play in this system. Our goal is, therefore, to undertake a series of electrophysiological studies in rat brain slices to elucidate the effects of kainate receptor activation on glutamatergic and GABAergic neurotransmission.

REPORTABLE OUTCOMES

- Kane-Jackson, R. and **Y. Smith** (2003) Pre-synaptic kainate receptors in GABAergic and glutamatergic axon terminals in the monkey globus pallidus. Neuroscience (in press).
- Charara, A., M. Paquet, J.D. Rothstein and **Y. Smith** (2002) Subcellular and subsynaptic localization of glutamate transporters in the monkey basal ganglia. In IBAGS VI (AM Graybiel, MR DeLong and ST Kitai eds). Kluwer Academic Press: New York, pp. 599-613.
- Jin, XT and **Y. Smith** (2003) Ultrastructural localization and functions of kainate receptors in the rat globus pallidus. Soc for Neurosci Abstr (in press)
- **Smith, Y.**, D. Raju and K.J. Ciombor (2003) Subcellular localization of neuronal and glial glutamate transporters in the monkey striatopallidal complex. Soc. for Neurosci. Abstr (in press).
- Kane-Jackson, R. and **Y. Smith** (2002) Pre- and post-synaptic kainate receptors in the monkey globus pallidus. Soc. for Neurosci. Abstr 28: 359.16.
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